aberrant receptor, prepared from a cell of a mammal suffering from a disease caused by the aberrant receptor, with a gene encoding the non-aberrant receptor, prepared from a cell of a mammal of the same species that does not carry the aberrant receptor.

24. (twice amended) A method of screening for a substance capable of causing an aberrant receptor, which has substantially changed affinity for substances, to operate in a manner similar to a non-aberrant receptor comprising: (1)expressing in a cell the gene encoding the aberrant receptor, (2) isolating the aberrant receptor from the cell, (3) providing a substance to the aberrant receptor and, (4) determining the operation activity of said substance on said receptor.

REMARKS

In the above referenced application, claims 14, 16-19, 21-24 and 26 are pending in the above referenced application and stand rejected. Claims 14, 16, 19, 21, 22-24 have been amended to more particularly set forth and distinctly claim the present invention. Support for the amendments is found throughout the specification. In light of the above amendments and following discussion, Applicant respectfully requests that the outstanding rejections be withdrawn and the claims be allowed.

A petition for an extension of time of three (3) months for responding to the outstanding Office Action and the appropriate fee authorization is enclosed herewith.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is entitled "VERSIONS WITH MARKINGS TO SHOW CHANGES MADE."

Applicant acknowledges and appreciates the Examiner's withdrawal of the rejection of claims under §112, paragraph 1 and 2 (except as described below) in view of the previous amendment.

The Examiner has not entered claims 14 and 21 due to typographic errors in the claims. Applicant respectfully submits that these minor errors are corrected by the above amendments to the claims.

Claims 22 and 23 stand rejected under 35 U.S.C. 112, first paragraph, for lack of enablement. Applicant traverses this rejection. Contrary to the Examiner's position, the activity of the receptor in the signal transduction system can be assayed by methods known in the art, e.g., by using a GTPγS binding assay. In this type of assay, a membrane fraction of cells, which express the G-protein coupled receptor is mixed with GTPγS and a ligand candidate or tissue extract, etc. The amount of GTPγS incorporated into the G protein is then measured, e.g., by liquid scintillation counting. The incorporation of G protein GTPγS occurs as a first step of the signal transduction system via a G-protein coupled receptor. Applicant has attached a journal article showing the use of this assay. See Ohtaki, et al., *J. Biol. Chem.*, (1999) Vol. 274, No. 52, p. 37041-37045. Thus, contrary to the Examiner's position, one of ordinary skill in the art would have known how to assay for activity of a receptor that is isolated from a cell and other components of the signal transduction system.

Applicant therefore, respectfully requests reconsideration and withdrawal of this rejection.

Claims 14, 16, 21, 24, 26 and dependent claims 18, 19, 22, 23 and 26 stand rejected under §112, paragraph 2 as indefinite. The Examiner is maintaining the rejection that these claims are indefinite because they recite the terms "operate", "operating" or "operation". The Examiner has stated that although these terms are described in the specification, they are not terms recognized in the art and thus, are indefinite. Applicant respectfully traverses this rejection. The test of indefiniteness is whether a person of ordinary skill in the art can understand the language of the claim when it is read in light of the specification. This is clearly the case here.

Applicant submits herewith copies of four abstracts of articles, which use the terms used in the claims. These abstracts demonstrate that a person skilled in the art

would have understood the meaning of the terms "operate", "operating" or "operation" as used in the claims, prior to the priority date of this application. (see 11: Brain Res. Mol. Brain Res., 2000, Dec. 28; 85 (1-2): 209-17; 7: J. Pharmacol. Exp. Ther., 2000, Dec.; 295 (3):1031-42; 45: Am. J. Physiol. Heart Circ. Physiol., 2000, Jun; 278(6):H2057-68; 60: Oncogene, 2000, Mar. 2;19(10):1354-61.) These terms as used in the claims are clearly definite to one of ordinary skill in the art.

Applicant therefore, respectfully requests reconsideration and withdrawal of this rejection.

The rejection of claims 16, 19 21, 23 and 26 in paragraphs 5.2-5.6 of the Office Action are obviated by the above amendments. Claim 26 was rejected as indefinite because it is not clear if the substance normally operates the wild type receptor or the aberrant receptor. In order to expedite prosecution of this case, claim 24, from which claim 26 depends, has been amended to recite that the aberrant receptor "has substantially changed affinity for substances." This amendment is supported at page 16, lines 12-19 of the specification. There is no inherent difference between the wild type receptor and the aberrant receptor, with regard to the operating substances. Thus, the functions of these receptors are intrinsically similar. However, the affinity of the wild type receptor for the substance, i.e., a natural ligand, is different from the affinity of the aberrant receptor for the natural ligand. Examples of aberrant receptors are receptors that cause disease due to the substantial change in the affinity for the natural ligand. (See e.g., page 16, lines 12-19).

Applicant respectfully requests reconsideration and withdrawal of this rejection.

The Examiner has maintained the rejection of claims 14-25 under U.S.C. 102(b) as being anticipated by Lebrun et al., The Journal of Biological Chemistry, Vol. 268, No. 15, pages 11272-11277, May 25, 1993. Applicant traverses this rejection. While the Applicant does not agree with the Examiner's position that Lebrun anticipates the present invention, Applicant respectfully submits that the amendment to claims 14,

16, 21, 22 and 24, which adds the phrase "which has substantially changed affinity for substances" further clarifies Applicant's invention.

Applicants therefore, respectfully request reconsideration and withdrawal of this rejection.

In view of the discussion above, it is respectfully submitted that the present application is in condition for allowance. An early reconsideration and notice of allowance are earnestly solicited. Should the Examiner wish to discuss the above amendment made herein, the undersigned attorney would appreciate the opportunity to do so. Thus the Examiner is hereby invited to call the undersigned, collect at the number shown below.

Respectfully submitted,

Date: April 3, 2001

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VERSIONS WITH MARKINGS TO SHOW CHANGES MADE.

In the Claims:

14. (twice amended) A method of screening substances for a substance capable of causing an aberrant receptor, which has substantially changed affinity for substances, to operate in a manner similar to a non-aberrant receptor comprising bringing ~~a~~ an aberrant receptor into contact with a subject substance and assaying the operation activity of said substance on said receptor.

16. (twice amended) A method of screening substances for a substance for treatment of a disease caused by an aberrant receptor, which has substantially changed affinity for substances, comprising bringing an aberrant receptor into contact with a substance and assaying the operation activity of said substance on said ~~product~~ receptor.

19. (twice amended) The screening method according to claim 16, wherein the method further comprises the step of selecting the receptor by comparing ~~a gene~~ the gene encoding the aberrant receptor, isolated from a cell of a mammal suffering from a disease caused by the aberrant receptor, with a gene encoding the non-aberrant receptor, prepared from a cell of a mammal of the same species that does not carry the aberrant receptor.

21. (amended) A method of preparing a substance for treatment of a disease caused by an aberrant receptor having a substantially changed affinity for substances, ~~which comprises~~ which results in the substantial reduction in activity of the signal transduction system of cells having the aberrant receptor, the method comprising:

selecting a substance from subject substances by bringing the aberrant receptor into contact with a subject substance, assaying the activity of said substance on the aberrant receptor,

selecting a substance that substantially operates the signal transduction system of the cell having the aberrant receptor wherein said activity is activity that restores wide-type activity of the receptor

and ~~preparing a substance judged to substantially operate the signal transduction system of a cell having the aberrant gene product wherein said activity is activity that restores wide-type activity to the receptor~~ admixing the selected substance with a pharmaceutically acceptable carrier.

22. (twice amended) The method according to claim 21, wherein the aberrant receptor, which has substantially changed affinity for substances, is isolated from a cell which expresses with the gene encoding the aberrant receptor.

23. (twice amended) The method according to claim 22, wherein the ~~method further comprises selecting~~ the gene encoding the aberrant receptor is selected by comparing ~~a~~ the gene encoding the aberrant receptor, prepared from a cell of a mammal suffering from a disease caused by the aberrant receptor, with a gene encoding the non-aberrant receptor, prepared from a cell of a mammal of the same species that does not carry the aberrant receptor.

24. (twice amended) A method of screening for a substance capable of causing an aberrant receptor, which has substantially changed affinity for substances, to operate in a manner similar to a non-aberrant receptor comprising: (1)expressing in a cell the gene encoding the aberrant receptor, (2) isolating the aberrant receptor from the cell, (3) providing a substance to the aberrant receptor and, (4) determining the operation activity of said substance on said receptor.

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Isolation and cDNA Cloning of a Novel Galanin-like Peptide (GALP) from Porcine Hypothalamus*

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Galanin is a widely distributed neuropeptide with a variety of physiological functions. Three galanin receptor subtypes, GALR1, GALR2, and GALR3, have been reported. We isolated a novel galanin-like peptide (GALP) from porcine hypothalamus by observing its activity for increasing [^{35}S]GTPγS binding to a membrane preparation of GALR2-transfected cells. The peptide had 60 amino acid residues and a non-amidated C terminus. The amino acid sequence of GALP-(9–21) was completely identical to that of galanin-(1–13). A cloned porcine GALP cDNA indicated that GALP was processed from a 120-amino acid GALP precursor protein. The structures of rat and human GALP-(1–60) were deduced from cloned cDNA, which indicated that the amino acid sequences 1–24 and 41–53 were highly conserved between humans, rats, and pigs. Receptor binding studies revealed that porcine GALP-(1–60) had a high affinity for the GALR2 receptor (IC_{50} = 0.24 nM) and a lower affinity for the GALR1 receptor (IC_{50} = 4.3 nM). In contrast, galanin showed high affinity for the GALR1 (IC_{50} = 0.097 nM) and GALR2 receptors (IC_{50} = 0.48 nM). GALP is therefore an endogenous ligand that preferentially binds the GALR2 receptor, whereas galanin is relatively non-selective.

Galanin, a C-terminally amidated peptide with 29 amino acid residues (non-amidated peptide with 30 residues in humans) was originally isolated from porcine intestine (1) and was later found to be ubiquitous in the central and peripheral nervous systems. It exerts diverse regulatory functions including central modulation of cognition, nociception, and feeding behavior, endocrine control of pituitary and pancreatic hormones, and regulation of gastrointestinal smooth muscle contractions (for review, see Refs. 2, 3).

Structurally, galanin is unrelated to any known family of neuropeptides or regulatory peptides, suggesting the presence of unknown members of a galanin peptide family. Indeed, the existence of a galanin-like peptide(s) in mammalian tissues has been proposed for several reasons. First, molecular heterogeneity of galanin-like immunoreactivity has been reported by several groups. Rökaeus *et al.* (4) first reported that rat brain and ileum contained galanin-like peptides that cross-reacted with galanin antiserum but differed from galanin in chromatographic characterization. Nevertheless, some of these immunoreactivities may be the result of galanin precursors or galanin-derived peptides, as described in the subsequent studies (5). Recently, Wang *et al.* (6) re-evaluated the existence of a novel galanin-like peptide in rat islets by showing the presence of galanin-like immunoreactivity that cross-reacted with antibody against porcine galanin but did not with antibody against rat galanin. Second, the physiological function of the three galanin receptor subtypes, GALR1[1] (7, 8), GALR2 (9, 10), and GALR3 (11, 12), is unlikely to be solely the distribution of different signals to target cells. The low affinity of human galanin for human GALR3 (12) suggests the possibility that these receptor subtypes are provided for different ligands. Third, the chimeric peptides antagonizing galanin *in vivo* were agonists for the cloned GALR1, GALR2 (10), and GALR3 (12), which implies the involvement of an unknown galanin receptor that is antagonized by the chimeric peptides. Another explanation is that the chimeric peptides elicit activation of an unknown receptor, which results in antagonizing the galanin effect. Endogenous ligand for such receptors must be structurally related to galanin.

In the present study, we studied endogenous galanin-like peptides using cloned GALR1 and GALR2 and discovered a novel galanin-like peptide, GALP, in the porcine hypothalamus. The physiological significance of GALP in light of the above discussion should be elucidated in future studies.

EXPERIMENTAL PROCEDURES

GALR1- and GALR2-expressing Cells—Rat GALR1 (8) and GALR2 (9, 10) cDNA were cloned using the PCR method. The cloned cDNA was expressed in CHO/*dhfr*$^-$ cells using a pAKKO-111 or pAKKO-1.11H mammalian expression vector (13).

GTPγS Binding Assays—Membrane fractions of the GALR1- and GALR2-expressing cells were prepared as described elsewhere (14) and were diluted to 12 (GALR1) or 20 μg/ml (GALR2) with GTPγS buffer (pH 7.4) containing 50 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1 μM GDP, and 1 mg/ml BSA. The membranes (200 μl) were mixed with 50 nM [^{35}S]GTPγS (NEN Life Science Products) (2 μl) and peptide samples (2 μl of dimethyl sulfoxide solution). After incubation at 25 °C for 60 min, the reaction mixtures were diluted with 1.5 ml of chilled TEM buffer, pH 7.4, containing 20 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% CHAPS, and 1 mg/ml BSA, and were then filtered through GF/F glass fiber

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF188490, AF188491, AF188492, and AF188493.

[1] The abbreviations used are: GALR1/2/3, galanin receptor 1/2/3; GALP, galanin-like peptide; PCR, polymerase chain reaction; CHO cells, Chinese hamster ovary cells; GTPγS, guanosine-5′-*O*-3-thiophosphate; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; HPLC, high pressure liquid chromatography; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; bp, base pair(s); ORF, open reading frame; RACE, rapid amplification of cDNA ends; ^{125}I-rat galanin, ^{125}I-labeled rat galanin.

filters. The filters were washed with 1.5 ml of TEM buffer, dried, and subjected to liquid scintillation counting. Dose-response curves were obtained with 3.7 μg/ml GALR1 or 7.5 μg/ml GALR2 membranes.

Preparation of Crude Extract from Porcine Hypothalamus—For a single batch of preparation, 30 porcine hypothalami including surrounding tissues (1 kg) were heat-denatured in boiling water (4000 ml) for 10 min, cooled on ice, and homogenized using a Polytron homogenizer. The homogenate was mixed with 1/17 volume of glacial acetic acid, stirred overnight at 4 °C, and centrifuged for 30 min at 10,000 rpm in a Hitachi RR10 rotor. Two volumes of acetone was added to the resultant supernatant for protein precipitation. After centrifugation to remove the precipitate, the clear extract was collected, concentrated using a rotary evaporator, and then phase-partitioned twice with diethyl ether. The aqueous extract from each of the two batches of preparation was loaded onto a C-18 column (YMCgel ODS-AM 120-S50, 50 × 200 mm), eluted with 60% acetonitrile-0.1% trifluoroacetic acid, and subjected to evaporation and lyophilization. The lyophilized powder (1–1.5 g from two batches of preparation) was dissolved in 50 ml of 0.1% trifluoroacetic acid, and each 10 ml was purified using an ODS80-TM HPLC column (21.5 × 300 mm, Tosoh). Elution was performed by a linear gradient increase of acetonitrile concentration from 20 to 60% in 0.1% trifluoroacetic acid for 120 min at a flow rate of 4 ml/min at 40 °C. The eluate was collected at each 8-ml fraction.

Isolation of Porcine GALP—The ODS80-TM HPLC fractions 45–48 from eight batches of preparation were lyophilized, dissolved in 1 M acetic acid, and loaded onto an SP-Sephadex C-25 (Amersham Pharmacia Biotech) column (2.1 × 4.5 cm). After washing the column with 1 M acetic acid and 2 M pyridine, GALP activity was eluted with 2 M pyridine/acetic acid. The eluate was lyophilized, dissolved in 1 M acetic acid, and gel-filtered on a Sephadex G50 (fine grade, Amersham Pharmacia Biotech) column (2.5 × 200 mm), which was equilibrated with 1 M acetic acid. The fractions with GALP activity were lyophilized, dissolved in 10 mM ammonium formate-40% acetonitrile buffer, and purified using a CM-2SW HPLC column (4.8 × 300 mm, Tosoh). Elution was performed by a linear gradient increase of ammonium formate concentration from 10 to 500 mM for 60 min at a flow rate of 1 ml/min at 25 °C. The eluate was collected at each 0.5-ml fraction. The active fractions-(94–96) were lyophilized, dissolved in 0.1% trifluoroacetic acid, and purified using a Super-Phenyl HPLC column (4.6 × 100 mm, Tosoh). Peptides were eluted with a linear gradient of acetonitrile concentration from 27 to 33% for 60 min in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min at 40 °C and collected at each 0.5-ml fraction. The active fractions-(66–68) were directly injected to a Super-ODS HPLC column (4.6 × 100 mm, Tosoh). Peptides were eluted with a linear gradient of acetonitrile concentration from 33 to 48% in 0.1% heptafluorobutyric acid for 60 min at a flow rate of 1 ml/min at 40 °C. The eluate was collected at each 0.5-ml fraction. The active fractions-(82–83) were pooled as purified peptide.

Peptide Sequencing—Four chymotryptic fragments were generated by incubating a purified peptide (50–100 pmol) with 10 pmol of TLCK-chymotrypsin (Sigma) in 1% NH_4HCO_3 buffer including 4% acetonitrile and 20% dimethyl sulfoxide. Fragment peptides were purified using a Spheri-5 RP-18 HPLC column (Brownlee, 2.1 × 30 mm) by a linear gradient elution of acetonitrile concentration from 0 to 70% for 30 min in 0.1% trifluoroacetic acid at a flow rate of 0.3 ml/min. The peptides were then subjected to N-terminal sequencing using a peptide sequencer (Biosystems Procise 491cLC, Perkin-Elmer) and to mass spectrometry using a JEOL HX-110 equipped with a cesium gun for the LSIMS mode. Undigested peptide was also subjected to N-terminal sequencing to determine the alignment of the fragment peptides.

Cloning of Porcine GALP cDNA—All oligonucleotides were custom synthesized by Japan Bio Service Co. Ltd. The porcine GALP cDNA fragment was amplified using the nested PCR method with degenerate primers. The first PCR was performed with 5′-CA(C/T)(A/C)GNGGI(A-/C)GNGGIGG(G/C)TGGAC-3′ (pGAL4-7F designed from peptide sequence HRGRGGWT); 5′-ATICCNAGIGCNGT(C/T)TTICC(C/T)TT-3′ (pGAL34-1R designed from peptide sequence KGKTALGI); *Taq* DNA polymerase (TaKaRa); and first-strand cDNA synthesized from porcine brain poly(A)$^+$ RNA by 32 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 4 min. The nested PCR was performed with 5′-GG(A/T/C)TGGACNCTNAA(C/T)AG(C/T)GC-3′ (pGAL9-3F designed from peptide sequence GWTLNSA), pGAL34-1R, *Taq* DNA polymerase (TaKaRa), and the first-PCR product as a template using the same thermal cycling profile as listed above. The resultant 98-bp DNA fragment (5′-GGCTGGACTTTAAATAGTGCTG-GTTACCTCCTGGGTCCCGTACTCCATCCGCCCTCCAGGGCTGAA-GGAGGCGGGAAGGGCAAAACAGCC CTGGGCAT-3′) was cloned and used as a probe for screening GALP cDNA in a porcine brain cDNA library (2.2 × 10^6 plaque-forming units, constructed using ZAP-cDNA Gigapack III Gold cloning kit, Stratagene). Phage DNA was transferred to nylon membranes, prehybridized in a hybridization buffer consisting of 5× SSPE (saline/sodium phosphate/EDTA), 5× Denhardt's solution, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA at 60 °C for 24 h, and then hybridized with the ^{32}P-labeled probes (5 × 10^5 cpm/ml) in the hybridization buffer at 60 °C for 24 h. The membranes were washed with 0.1% SDS-0.2× SSC at 50 °C for 30 min and subjected to autoradiography with x-ray films (Kodak Biomax film) at −70 °C for 3 days. Five positive clones were isolated by plaque purification. The plasmids were excised using a Rapid Excision kit (Stratagene).

Cloning of Rat GALP cDNA—Rat GALP cDNA was screened from a rat brain cDNA library (2.2 × 10^6 plaque-forming units, constructed using the ZAP-cDNA Gigapack III Gold cloning kit, Stratagene) using a 356-bp porcine GALP ORF cDNA probe, which was made using PCR with a forward primer (5′-ATGGCTCTGACTGTCCCTCTGATCGT-TCT-3′) and a reverse primer (5′-TGAAACCTCGTAGTTCCTGGTCG-GATTCG-3′). Hybridization was performed as described above. The membranes were washed with 0.1% SDS-2× SSC at 50 °C for 30 min. Two positive clones were isolated to a single plaque.

Cloning of Human GALP cDNA—A human GALP cDNA fragment was amplified from human whole brain cDNA using *Taq* DNA polymerase (TaKaRa), a forward primer (5′-AGGCTGGACCCTCAATAGT-GCTGGTTAC-3′ (F/h120)), and a reverse primer (5′-CCATCTATGGC-CTTCCACAGGTCTAGGA-3′ (R/h120)) by the PCR program as 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 10 min. The sequence of the resultant 126-bp DNA was obtained, and two primers (5′-CAAATGGGTGACCAAGACG-GAAAGAGGG-3′ (1F/h120) and 5′-GGTCTAGGATCTCAAGGGCT-GTCTCCCT-3′ (1R/h120)) were designed for 5′-RACE and 3′-RACE experiments. For the 3′-RACE experiment, PCR was performed first with human cDNA (CLONTECH, Marathon-Ready cDNA), F/h120 primer, and AP1 primer (CLONTECH) and then with the first-PCR product, 1F/h120 primer, and AP2 primer (CLONTECH). Both reactions were done with *Taq* DNA polymerase (TaKaRa) using the touch down program: 5 cycles of 94 °C for 30 s and 72 °C for 2 min, 5 cycles of 94 °C for 30 s and 70 °C for 2 min, 25 cycles of 94 °C for 20 s and 68 °C for 2 min, with a final extension at 68 °C for 10 min. The 5′-RACE experiment was performed as described above, first with the R/h120 and AP1 primers and then with the 1R/h120 and AP2 primers. A 473-bp DNA was amplified from human whole brain cDNA (CLONTECH) using *Pfu* DNA polymerase (Stratagene), a forward primer (5′-GAG-GAGCCAGAGAGAGCTGCGGAGAG-3′ (1F/hORF)), and a reverse primer (5′-GAGCTGGAGAAGAAGGATAGGAACAGGG-3′ (3R/hORF)) by the PCR program as 35 cycles of 94 °C for 30 s and 70 °C for 5 min, with a final extension at 72 °C for 10 min.

DNA Sequencing—The sequencing reactions were performed using BigDye terminator cycle sequencing FS ready reaction kit (Perkin-Elmer). The reaction mixtures were analyzed using an automated sequencer (Perkin-Elmer, Applied Biosystems Prism 377).

Peptide Synthesis—Porcine GALP-(1–60) was synthesized by a peptide synthesizer (Applied Biosystems, model 430A). The synthesized peptide was de-protected using HF and purified to a single peak. The sequence of the synthesized peptide was confirmed by sequencing analysis and mass spectrometry after digestion into four chymotryptic peptide fragments.

Receptor Binding Experiments—The experiment was performed using membranes from the CHO cell transfectants. Membranes (2.9 μg/ml for GALR1 and 6 μg/ml for GALR2) were incubated with 100 pM ^{125}I-rat galanin (NEN Life Science Products) and increasing concentrations of rat galanin (the Peptide Institute) or porcine GALP (1–60) at 25 °C for 90 min in binding buffer (pH 7.3) containing 20 mM Tris, 2.5 mM magnesium acetate, 2 mM EGTA, 0.5 mM *o*-phenanthroline, 0.5 mM phenylmethylsulfonyl fluoride, 20 μg/ml lepeptin, 10 μg/ml pepstatin, 8 μg/ml E-64, and 1 mg/ml BSA. The reaction mixtures were diluted with 1.5 ml of TEM buffer and filtered through GF/F filters pretreated with 0.3% polyethyleneimine. The filters were washed with 1.5 ml of TEM buffer and subjected to γ-counting.

RESULTS AND DISCUSSION

We established stable CHO cell transfectants expressing a large number of rat GALR1 (13.8 pmol/mg protein) and GALR2 (6.6 pmol/mg protein). These cells allowed simple and sensitive detection of galanin-like agonistic activity by measuring the increase in [^{35}S]GTPγS binding to cell membranes. Using this assay combined with C-18 reversed-phase HPLC analysis, we investigated the galanin-like agonistic activity found in porcine

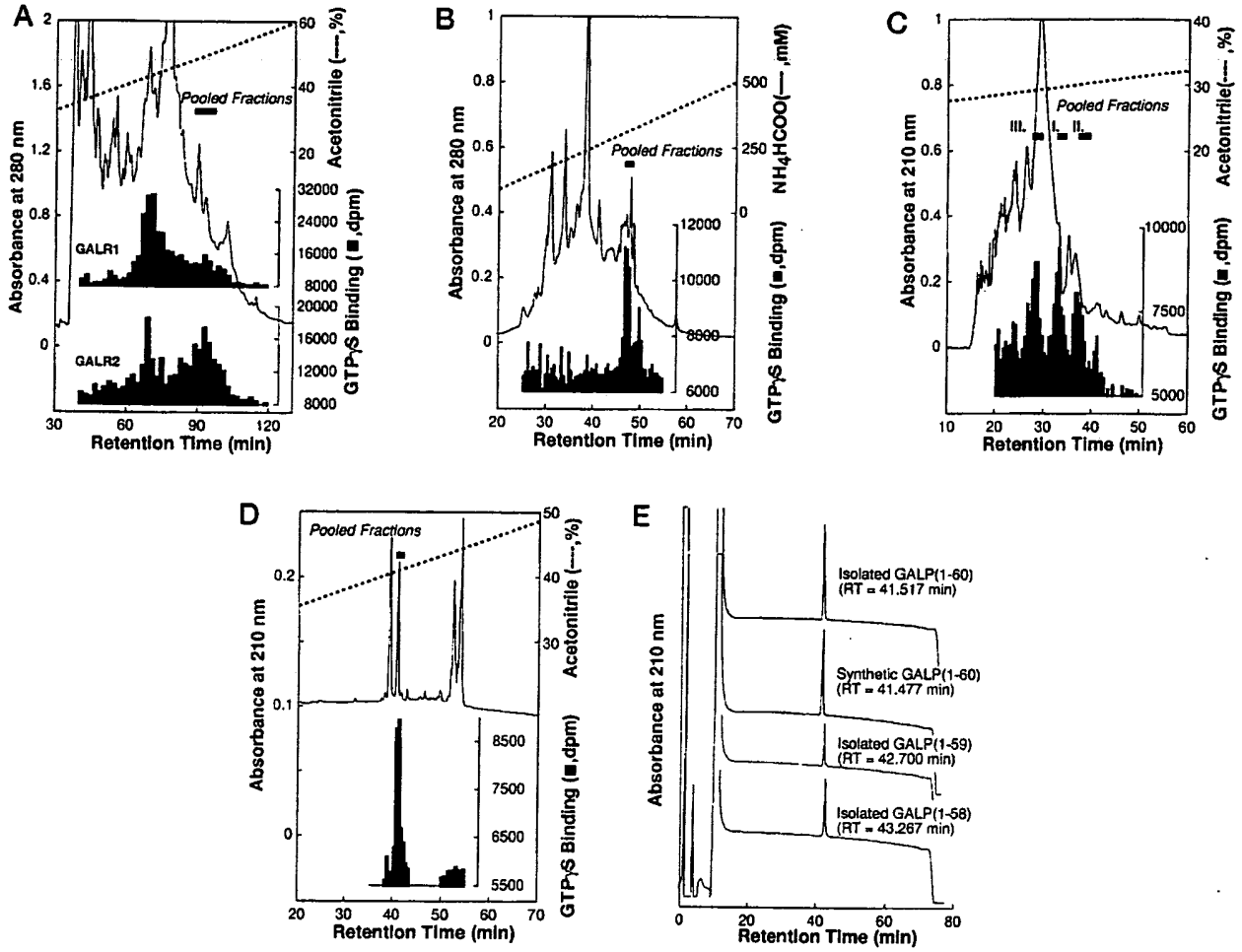

FIG. 1. **Isolation of GALP from porcine hypothalamus.** *A–D*, elution profiles of an ODS80-TM HPLC column (*A*), a CM-2SW HPLC column (*B*), a Super-Phenyl HPLC column (*C*), and a Super-ODS HPLC column (*D*). [^{35}S]GTPγS binding assays were performed using membranes from the GALR1 (*A*) and GALR2 (*A–D*) transfectants. *E*, purified and synthetic peptides were analyzed under the same conditions as described for *D*.

hypothalamus. As shown in Fig. 1*A*, a single major peak of activity was observed for GALR1. This activity was attributed to galanin because of its elution profile and immunoreactivity. In contrast, a second peak following the first galanin peak was observed for GALR2. A preliminary characterization of this activity revealed that it was broken by Pronase treatment, was more cationic and larger than galanin (M_r = 5000–6000), and was undetectable using a porcine galanin radioimmunoassay kit (Peninsula). Galanin-derived peptides with a similar elution profile were not found in a previous detailed study describing variant forms of galanin in porcine brains (5). We therefore considered that the porcine hypothalamus contains a non-galanin-derived peptide with GALR2-preferential agonistic activity. Similar GALR2-agonistic activity was also found in extract from other porcine tissues including the pituitary gland, brain, and small intestine.

The GALR2-agonistic activity was isolated from porcine hypothalamus for further characterization. The activity was separated into major activity I, minor activity II, and minor activity III during the Super-Phenyl HPLC step (Fig. 1*C*). The major activity I was further purified to a single peak (Fig. 1*E*). Approximately 200 pmol of the purified peptide was obtained from the dissected hypothalamus of 240 brains (8–9 kg of wet tissues). The N-terminal 30 amino acid residues of the peptide were determined as APVHRGRGGWTLNSAGYLLGPVLHPPSRAE by direct N-terminal sequencing analysis. The peptide was further digested into four peptide fragments with chymotrypsin. The sequences of these fragments were determined by N-terminal sequencing and mass spectrometry as follows: TLNSAGY (*m/z* = 725.5), APVHRGRGGW (*m/z* = 1092.5), KAIDGLPYPQSQLAS (*m/z* = 1588.6), and LLGPVLHPPSRAEGGGKGKTALGILD(LW/HY) (*m/z* = 2854.0). The C-terminal 2 residues of the last fragment could not be determined by peptide sequencing. Two possible sequences (LW/HY) were deduced from the *m/z* value and substrate specificity of chymotrypsin. Taking together the results from the direct N-terminal sequencing analysis and the chymotryptic fragments, the amino acid sequence of the purified peptide was determined as APVHRGRGGWTLNSAGYLLGPVLHPPSRAEGGGKGKTALGILD(LW/HY)KAIDGLPYPQSQLAS. The C terminus was not amidated, in contrast to many other neuropeptides. A thorough data base search revealed that the 60-amino acid sequence was indeed novel. It was of particular interest that 13 amino acid residues of the peptide-(9–21) were completely identical to the N-terminal 13 residues of galanin. The N-terminal 15 residues of galanin are conserved in numerous species (2, 3). Furthermore, these 13 residues are considered to be highly involved in galanin receptor binding and are used as a core sequence for producing chimeric peptide antagonists such as galanin-(1–13)/substance P-(5–11) amide (M15), galanin-(1–13)/spantide I (C7), and galanin-(1–13)/bradykinin-(2–9) amide (M35). Because of its striking structural charac-

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                                    TTCAGCCTCAAGCACCCATCCCTCCAGCCCTCAG      34

ATGGCTCTGACTGTCCCTCTGATCGTTCTTGCAGTCCTGCTCAGCCTGATGGAG                     89
MetAlaLeuThrValProLeuIleValLeuAlaValLeuLeuSerLeuMetGlu                     19
                ▼
TCTCCAGCCTCTGCTCCGGTCCACAGGGGGCGAGGAGGCTGGACCCTCAACAGT                     142
SerProAlaSerAlaProValHisArgGlyArgGlyTrpThrLeuAsnSer                        36

GCTGGTTACCTCCTGGGTCCCGTACTCCATCCGCCCTCCAGGGCTGAAGGAGGC                     196
AlaGlyTyrLeuLeuGlyProValLeuHisProProSerArgAlaGluGlyGly                     54

GGGAAGGGGAAGACAGCCCTCGGGATCCTGGACCTGTGGAAGGCCATTGATGGG                     250
GlyLysGlyLysThrAlaLeuGlyIleLeuAspLeuTrpLysAlaIleAspGly                     72
                ▼
CTCCCCTATCCCCAGTCTCAGTTGGCCTCCAAGAGGAGTCTGGGGGAGACTTTC                     304
LeuProTyrProGlnSerGlnLeuAlaSerLysArgSerLeuGlyGluThrPhe                     90

GCCAAACCAGACTCTGGAGTAACATTTGTTGGAGTTCCTGACGTGGTGCCGTGG                     358
AlaLysProAspSerGlyValThrPheValGlyValProAspValValProTrp                     108

AAACGAATCCGACCAGGAACTACGAGGTTTCAGATCTAGGCAAGCTCTGCAAGA                     412
LysArgIleArgProGlyThrThrArgPheGlnIle***                                    120

ACGTTCCAAAGGAGAAAGATGCCTTGCCGTCATATATGCCTCCAAACTTCCGCT                     466
CCAAACTTCCCCCCCCGTCTCCAGATCCTCCTGAAACCCTAGGTAGACACCCTCT                    520
ACTGAGACTGGGAGCCTGAAAGTAAATCCCCAAATCCCAGGTAGAAAATGGGGA                     574
GCATTTGAAGAATTATTCTCAAAAGTCCCCGGACTGTGCCAGGTTTCACTGATC                     628
CCCCCCTCCCCCTTGGACTAAGTGTAAAGCGATGTAAACCAACTCAAGAATAAT                     682
TCTGAAACCATTCAGGAGATCCGGAGAGGAATCGGGAAATACTCCTGCAGTGCA                     736
TTTAAAGTAACTGGGTCCTATGCAACATGAGCCATTGGATCATACAATATTGAT                     790
ATCCCTTCTAACACGGAGGTTCTAGGGTGTCTCAGCTGGAAAAGATTCTTCAGA                     844
GTAGCATGCTTGCCTTACCCCATCCTTCTCACCCCACCCCGAGCCTCCTCCAGC                     898
AGAAAGGACGAGAGGCCATCTGGAGCAGAGCAGAGAGAATAAATATTCCCTTTC                     952
AAAGAAAAAAAAAAAAAAAAA                                                      974
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FIG. 2. **DNA sequence and deduced amino acid sequence of porcine GALP.** *Heavy underline*, galanin/GALP-shared 13 residues; ▼, N- and C-terminal processing sites; *underline*, polyadenylation signals. The DNA sequence is available from the GenBank™ (accession no. AF188490).

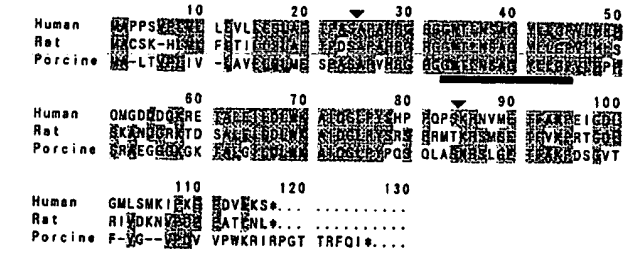

FIG. 3. **Amino acid sequence comparison of human, rat, and porcine GALP.** *Shaded characters*, conserved residues; *solid underline*, galanin/GALP-shared 13 residues; ▼, N- and C-terminal processing sites.

TABLE I
Pharmacological characterization of GALP

Mean values ± S.E. of 6–9 determinations from 2–3 different experiments (receptor binding) or those of 4 determinations from 2 different experiments (GTPγS binding) are shown.

	Receptor binding (IC_{50})		[^{35}S]GTPγS binding (EC_{50})	
	GALR1	GALR2	GALR1	GALR2
	nM		*nM*	
Rat galanin	0.097 ± 0.004	0.48 ± 0.02	0.16 ± 0.02	5.2 ± 0.5
Porcine GALP	4.3 ± 0.09	0.24 ± 0.01	30 ± 4	2.4 ± 0.4

teristic, the peptide was designated GALP-(1–60). Two minor components of GALP activity (Fig. 1*E*) were also purified from minor activity II (Fig. 1*C*) and were determined to be GALP-(1–59) and GALP-(1–58).

Molecular cloning of porcine GALP cDNA was performed by library screening using a 98-bp DNA probe obtained by degenerated PCR. We isolated a 974-bp cDNA clone (GenBank™ accession no. AF188490) that had a 360-bp ORF starting from ATG at position 35 (Fig. 2). The adjacent sequence of this ATG (GCCCTCAGatgG) partially conformed to Kozak's rules (GCCACCatgG) (15). Searching the upstream 5′ non-coding region of other clones, we found an in-frame stop codon and no initiation codon (data not shown). An AATAAA polyadenylation signal was found in the 3′ non-coding region of this clone. The open reading frame encodes the GALP precursor protein with 120 amino acid residues. The N-terminal 20–22 residues of the precursor protein showed characteristics of a signal sequence consisting of hydrophobic clusters followed by small polar residues. Computer analysis using the SignalP server (16) predicted that the most likely cleavage site of the signal peptide was between Ala^{23} and Pro^{24}. This was very close to the experimental result in that the purified peptide started from Ala^{23}. Therefore, the mature peptide directly flanks the signal peptide. This is different from the case of galanin in which the N terminus is generated by successive cleavage of a signal peptide and processing at paired basic Lys-Arg residues (17–19). The C terminus of GALP-(1–60) was revealed to be generated by cleavage at the residues Ser^{82}-Lys^{83}-Arg^{84}. This C-terminal cleavage site is similar to that of human galanin (Ser-Lys-Arg) (17) or that of glucagon (Thr-Lys-Arg) (20), which provides a non-amidated serine or threonine residue at the C terminus. The two residues, which were not clear during peptide sequencing, were determined to be Leu^{66}-Trp^{67}.

We next isolated a rat GALP cDNA clone from a cDNA library, which had a 369-bp open reading frame (GenBank™ accession no. AF188491). Given Kozak's rule, however, the second ATG (TCCAGGatgG), and not the first ATG (AGCTGTatgC), serves as an initiation codon. The deduced rat GALP precursor protein had 115 amino acid residues. A human GALP cDNA clone (GenBank™ accession no. AF188492) was further obtained using PCR. It had an open reading frame encoding the human GALP precursor protein of 116 amino acid residues. The alignment of human, rat, and porcine precursor proteins (Fig. 3) suggests that human and rat mature peptides start from Ala^{25} and Ala^{24}, respectively. Processing at the C terminus probably occurs at Ser^{84}-Lys^{85}-Arg^{86} in humans and at Thr^{83}-Lys^{84}-Arg^{85} in rats, which corresponds to the Ser^{82}-Lys^{83}-Arg^{84} cleavage site of porcine GALP. Consequently, human and rat GALP-(1–60) will be produced. The additional paired basic residues, Gly^{57}-Lys^{58}-Arg^{59}, were found in human GALP-(1–60). Nevertheless, processing into a smaller peptide at this site is controversial because this site is not conserved in rats and pigs. The amino acid sequence of GALP-(1–60) was conserved at residues 1–24 and 41–53 between humans, rats, and pigs. Interestingly, DNA sequences for the galanin/GALP-shared 13 residues were more conserved among the GALP of different species than between the GALP and galanin (17–19) of the same species, which indicates that GALP is not a splicing variant of galanin and that the genes for the two peptides diverged early in the process of evolution.

Porcine GALP-(1–60) was chemically synthesized for pharmacological characterization and was shown to have the same retention time as the purified natural peptide GALP-(1–60) (Fig. 1*E*). Receptor binding studies were performed using membrane preparations of the CHO transfectants expressing rat GALR1 and GALR2. The K_d values of ^{125}I-rat galanin from the saturation binding experiment were 18 pM for GALR1 and 65 pM for GALR2. The IC_{50} values of rat galanin and porcine GALP-(1–60) were determined by competitive binding experiments using ^{125}I-rat galanin. As summarized in Table I, rat galanin had quite a high affinity for GALR1, whereas porcine GALP-(1–60) had a 44-fold lower affinity. GALP-(1–60) and galanin had similarly high affinities for GALR2. The agonistic activity of GALP and galanin was measured using a [^{35}S]GTPγS binding assay, and the EC_{50} values were obtained from dose-response curves. Rat galanin exerted potent activity for GALR1, whereas porcine GALP-(1–60) was 180-fold less potent. In contrast, porcine GALP-(1–60) and rat galanin

showed a similar activity for GALR2.

In summary, we discovered a novel galanin-like peptide, GALP, in porcine hypothalamus that preferentially binds and activates the GALR2 relative to the GALR1. The binding affinity of GALP for GALR3 was not determined, because significant ^{125}I-rat galanin binding was not reproduced with GALR3-transfected COS-7 cells. Both galanin and GALP are distributed in porcine hypothalamus, whereas all three subtypes of the galanin receptor have been found in the rat hypothalamus (8, 21). To elucidate the physiological significance of GALP in the hypothalamus, immunohistochemical studies for revealing the localization of GALP in comparison with that of galanin and its receptors will be required. The possible presence of GALP-selective binding sites should also be investigated. It is our hope that the discovery of GALP will contribute to the development of a new horizon of knowledge about neuroendocrine regulation.

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Distribution of dopamine D1 receptors in the nucleus paraventricularis of the hypothalamus in rats: an immunohistochemical study.

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The present study investigated the distribution of dopamine D1 receptor protein in the nucleus paraventricularis of the hypothalamus. It was found that the nucleus paraventricularis of the hypothalamus contains a relatively large number of cells which are positive for presence of dopamine D1 receptor protein. The vast majority of dopamine D1 receptor-positive neurons was found in the magnocellular part, but they were also present in considerable quantity in the parvocellular part of this subregion of the hypothalamus. When measured by the Western blot technique, the quantity of D1 receptor protein found in the paraventricular nucleus of the hypothalamus was at the level found in the prefrontal cortex. It was also found that dopamine D1 receptor protein was present in neurons constitutively displaying phosphorylated CREB protein, i.e. neurons which are, as might be speculated, under the tonic influence of neurotransmitters whose receptors operate via cAMP and pCREB as second or third messengers. The presence of dopamine D1 receptors in the nucleus paraventricularis of the hypothalamus may suggest, at an anatomical level, that these receptors are involved in controlling the release of hormones, as well as their synthesis at the level of transcription, which is regulated by phosphorylation of CREB protein. Finally, the present immunocytochemical findings offer an anatomical substrate for the role of dopamine and its receptors of D1 subtype in the regulation of the activity of paraventricular

neurons seen in the functional studies.

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Dose- and time-dependent bimodal effects of kappa-opioid agonists on locomotor activity in mice.

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The kappa-opioid agonists U50488H, bremazocine, and BRL52537, and the mu-opioid agonist morphine were compared in their ability to modify spontaneous motor activity in male NMRI mice. Higher, analgesic doses of the kappa-agonists reduced rearing, motility, and locomotion in nonhabituated mice. These effects, as well as the analgesic action of U50488H, were blocked by the selective kappa-opioid antagonists nor-binaltorphimine and DIPPA. In contrast, lower, subanalgesic doses (1.25 and 2.5 mg/kg for U50488H; 0.15 and 0.075 mg/kg for bremazocine, and 0.1 mg/kg for BRL52537) time dependently increased motor activity. The stimulatory effects of U50488H and bremazocine were not observed in habituated animals and were reduced by dopamine depletion. Surprisingly, the stimulatory effects of U50488H and bremazocine were not blocked by nor-binaltorphimine and DIPPA but they were completely eliminated by naloxone (0.1 mg/kg). The effects of morphine were dose-dependent; an initial limited suppression was followed by increased motility and locomotion (but not rearing) with a peak effect at 20 mg/kg both in habituated and nonhabituated mice. The selective mu-opioid antagonist beta-funaltrexamine blocked morphine-induced motor stimulation and analgesia but failed to affect the analgesic and motor stimulatory effects of U50488H. The results indicate that kappa-opioid agonists interact with different functional subtypes of opioid receptors. A stimulatory, naloxone-sensitive but nor-binaltorphimine- and DIPPA-insensitive subtype of opioid

receptor appears to operate only when the dopamine system is tonically active in nonhabituated animals. At higher doses, kappa-agonists produce analgesia and motor suppression, effects mediated by a "classic" (inhibitory) kappa-opioid receptor.

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Differential role of ionotropic glutamatergic mechanisms in responses to NTS P(2x) and A(2a) receptor stimulation.

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Activation of ATP P(2x) receptors in the subpostremal nucleus tractus solitarii (NTS) via microinjection of alpha,beta-methylene ATP (alpha,beta-MeATP) elicits fast initial depressor and sympathoinhibitory responses that are followed by slow, long-lasting inhibitory effects. Activation of NTS adenosine A(2a) receptors via microinjection of CGS-21680 elicits slow, long-lasting decreases in arterial pressure and renal sympathetic nerve activity (RSNA) and an increase in preganglionic adrenal sympathetic nerve activity (pre-ASNA). Both P(2x) and A(2a) receptors may operate via modulation of glutamate release from central neurons. We investigated whether intact glutamatergic transmission is necessary to mediate the responses to NTS P(2x) and A(2a) receptor stimulation. The hemodynamic and neural (RSNA and pre-ASNA) responses to microinjections of alpha,beta-MeATP (25 pmol/50 nl) and CGS-21680 (20 pmol/50 nl) were compared before and after pretreatment with kynurenate sodium (KYN; 4.4 nmol/100 nl) in chloralose-urethan-anesthetized male Sprague-Dawley rats. KYN virtually abolished the fast responses to alpha,beta-MeATP and tended to enhance the slow component of the neural responses. The depressor responses to CGS-21680 were mostly preserved after pretreatment with KYN, although the increase in pre-ASNA was reduced by one-half following the glutamatergic blockade. We conclude that the fast responses to stimulation of NTS P(2x) receptors are mediated via glutamatergic ionotropic mechanisms, whereas the slow responses to stimulation of NTS P(2x) and A(2a)

receptors are mediated mostly via other neuromodulatory mechanisms.

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Two mutations affecting conserved residues in the Met receptor operate via different mechanisms.

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We have investigated the mechanism by which two oncogenic mutations (M1268T and D1246H/N; Amino-acids are numbered according to Schmidt et al., 1999) affecting conserved residues in the catalytic domain of the Met receptor, activate its transforming potential. Both mutations were previously found in tumorigenic forms of the Ret and Kit receptors, respectively. The mutated residues are located either in the P+1 loop (M) or within the activation loop (A-loop) (D), which in a number of receptor tyrosine kinases harbors a pair of tandem tyrosines (Y1252-1253 in Met). Ligand-induced dimerization promotes their phosphorylation, and locks the A-loop into an open conformation. When unphosphorylated, the tandem tyrosines inhibit enzymatic activity by blocking the active site. Upon Y-->F mutation of Y1252-1253, neither ligand binding nor Tpr-mediated dimerization can release this block. Here we show that the M1268T mutation partially rescues the kinase activity (and the transforming ability) of the Y1252-1253F Tpr-Met mutant, but is completely dependent on dimerization for its effect. In contrast, the two D1246H/N mutants strictly depend on Y1252-1253 for activity. Surprisingly, however, they constitutively activate the isolated cytoplasmic TK domain of Met (Cyto-Met). These data indicate that the two mutations operate via distinct mechanisms.